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The antioxidative function of eicosapentaenoic acid in a marine bacterium, *Shewanella marinintestina* IK-1

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Abstract When the eicosapentaenoic acid (EPA)-deficient mutant strain IK-1Δ8 of the marine EPA-producing *Shewanella marinintestina* IK-1 was treated with various concentrations of hydrogen peroxide (H_2O_2), its colony-forming ability decreased more than that of the wild type. Protein carbonylation, induced by treating cells with 0.01 mM H_2O_2 under bacteriostatic conditions, was enhanced only in cells lacking EPA. The amount of cells recovered from the cultures was decreased more significantly by the presence of H_2O_2 for cells lacking EPA than for those producing EPA. Treatment of the cells with 0.1 mM H_2O_2 resulted in much lower intracellular concentrations of H_2O_2 being consistently detected in cells with EPA than in those without EPA. These results suggest that cellular EPA can directly protect cells against oxidative damage by shielding the entry of exogenously added H_2O_2 in *S. marinintestina* IK-1.

Keywords: Eicosapentaenoic acid; Hydrogen peroxide; Oxidative stress; Protein carbonyl; *Shewanella*

1. Introduction

Previously we showed that eicosapentaenoic acid (EPA) has the potential to prevent the entry of H_2O_2 through the cell membrane in *Escherichia coli* strains genetically modified to produce EPA. The *E. coli* strains DH5α [1] and UM2 [2] transformant cells, which contained EPA, became resistant to oxidative stress by exogenous H_2O_2. When the *E. coli* strains were transformed with the EPA biosynthesis genes (*pfaA*, *pfaB*, *pfaC*, *pfaD*, and *pfaE*) [3] from *Shewanella pneumatophori* SCRC-2738 [4], the transformant produced EPA at levels of approximately 10% of total fatty acids. In the case of *E. coli* strain DH5α, the colony-forming ability of EPA-containing cells treated with 3.0 mM H_2O_2 was similar to that of cells that were not exposed to H_2O_2. However, colony-forming ability was lost rapidly in cells without EPA under the same conditions. This was reflected in the difference in the degree of protein carbonylation between the strain with EPA and that without EPA. The treatment of both types of cells with H_2O_2 changed the amount of cells recovered from the cultures (estimated by the amount of fatty acid). The recovery of fatty acids was lower from cultures of *E. coli* cells without EPA than those with EPA, but it did not affect the fatty acid compositions. From these data, it has been suggested that cellular EPA is stable in vivo in the presence of H_2O_2 and may directly protect cells against oxidative damage. Using catalase-deficient *E. coli* UM2 that had been transformed with the same EPA biosynthesis genes, almost the same results were obtained [2], although the cells of *E. coli* UM2 were treated with 0.3 mM H_2O_2. In the case of *E. coli* UM2, cells were suspended in phosphate-buffered saline (PBS) and then treated with H_2O_2 under bacteriostatic conditions (medium-free and no agitation). Much lower intracellular concentrations of H_2O_2 were detected in cells with EPA than in those lacking EPA. From these results, it was suggested that cellular EPA could directly protect cells against oxidative damage by preventing the entry of exogenously added H_2O_2. A similar antioxidative mechanism is thought to operate in bacteria that produce EPA naturally. However, no evidence for this was found in the literature.

Shewanella marinintestina IK-1 is an EPA-producing bacterium that was isolated from a squid body [5]. Although EPA is regarded as a modulator of membrane fluidity at low temperature also in this bacterium [5], we present here the results of an investigation into the possible role of EPA in protecting against oxidative stress (exogenous addition of H_2O_2) in bacteria that inherently produce EPA. The study compared the responses against exogenously added H_2O_2 of *S. marinintestina* IK-1 and its EPA-deficient mutant (strain IK-1Δ8).

2. Materials and methods

2.1. Bacterial cells and cultivation

S. marinintestina IK-1 [5] and its EPA-deficient mutant *S. marinintestina* IK-1Δ8 (see below) were used as the test organisms throughout the work. Both strains were precultured by agitation at 180 rpm in Luria–Bertani (LB) medium containing 3.0% (w/v) NaCl normally at 15 °C. Non-growth cells of the both strains were prepared as follows. The cells were grown until the culture had an optical density (OD) of 1.0 at 600 nm (OD_{600}) and were harvested by centrifugation at 3500 × *g* for 5 min at 4 °C. Collected cells were washed three times with PBS containing 3.0% (w/v) NaCl and suspended in the same buffer at an OD_{600} of 1.0. The cell suspension was mixed with a solution of

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Abbreviations: CFU, colony-forming unit; EPA, eicosapentaenoic acid; H_2O_2+, in the presence of H_2O_2; H_2O_2−, in the absence of H_2O_2; LB, Luria–Bertani; OD, optical density; PBS, phosphate-buffered saline

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H_2O_2 (30% solution in water; Wako Chemicals, Osaka, Japan) normally at a final concentration of 0.01 mM and then incubated without agitation for 30 min at 15 °C. Cells were harvested at appropriate time intervals by centrifugation and used for further assay. The number of colony-forming units (CFUs) was measured as previously described [1].

2.2. Generation of EPA-deficient mutant of S. marinintestina IK-1

S. marinintestina IK-1 has an EPA biosynthesis gene cluster (N. Morita et al., unpublished results), of which structure is very similar to that from *S. pneumatophori* SCRC-2738. Namely, the EPA biosynthesis gene cluster of *S. marinintestina* IK-1 consisted of *pfaE*, *pfaA*, *pfaB*, *pfaC*, and *pfaD* in this order. To generate EPA-deficient mutants of *S. marinintestina* IK-1, insertional inactivation mutagenesis was performed targeting the *pfaD* gene. An insertional fragment of *pfaD* was amplified using primers of 5′-CGCTACCCAATGGCCCTTAC-3′ and 5′-GCTGGCTCCAGCTTCAACAC-3′ and cloned into the *Eco*RV site of mobilizable suicide pKNOCK-Cm [6]. The resulting *pfaD*: pKNOCK-Cm construct was introduced into *S. marinintestina* IK-1 from *E. coli* BW20767 (ATCC47084) by conjugal transfer as described by Fukuchi et al. [7]. Chloramphenicol-resistant exconjugants arose from plasmid integration into the *S. marinintestina* IK-1 chromosome in a single crossover event yielding strain IK-1Δ8 with *pfaD* insertionally inactivated. The site of plasmid insertion was verified by PCR amplification of a portion of the *pfaD* gene using primers located upstream and downstream of the insertion site.

2.3. Analytical procedures

The carbonyl content in oxidatively modified cellular proteins was determined using cells treated with 0.01 mM H_2O_2, as previously described [8]. The degree of carbonylation was expressed as nmol of protein carbonyls per mg of protein. Protein was assayed by the Bradford method [9].

The intracellular and extracellular concentrations of H_2O_2 were measured with the method by González-Flecha and Demple [10,11]. The cells of *S. marinintestina* strains IK-1 and IK-1Δ8 that had been suspended in PBS at an OD_{600} of 1.0, were treated with and without H_2O_2 and then separated as supernatants and cell pellets by centrifugation as previously described [2]. The extracellular concentration of H_2O_2 in the supernatants was measured using a titanium-based method [12]. The cell pellets were resuspended in the same buffer at the same concentration of cells and then allowed to stand for 30 min at 15 °C. This time is sufficient for the medium to reach the intracellular steady-state concentration of H_2O_2 on the basis of its free diffusion through the cell membrane [10]. After centrifugation the intracellular concentration of H_2O_2 was measured by assaying the concentration in the supernatants.

Packed wet bacterial cells from 10 ml of cultures were subjected to methanolysis using 2 M methanolic HCl in the presence of 20 μg of heneicosanoic acid as an internal standard, as previously described [13]. The resulting fatty acid methyl esters were analyzed by gas–liquid chromatography, as previously described [13]. The amount of fatty acids was expressed as micrograms of their methyl esters per millilitre of culture.

To measure the dry cell weight the packed cells were freeze-dried and weighed. Total lipids from dry cells were extracted by the Bligh and Dyer method [14]. Phospholipids were separated by one-dimensional thin-layer chromatography of total lipids. Fatty acid composition and phosphorus contents of individual phospholipids were determined, as described previously [15].

The catalase activity of their cell-free extracts of *S. marinintestina* strains IK-1 and IK-1Δ8 was measured spectrophotometrically at 240 nm at room temperature, as described previously [16].

3. Results

3.1. Effects of H_2O_2 on growth of S. marinintestina strains IK-1 and IK-1Δ8

S. marinintestina strains IK-1 and IK-1Δ8 similarly grew in LB medium containing NaCl at 15 °C (data not shown). A similar finding has been observed for EPA-producing *Photobacterium profundum* SS9 and its EPA-deficient mutant [17]. To investigate their susceptibility to H_2O_2, cells of these strains were subjected to bacteriostatic conditions, where the cells were suspended in PBS containing (H_2O_2+) or not containing (H_2O_2−) various concentrations of H_2O_2 and incubated for 30 min at 15 °C with no agitation. In Fig. 1 the number of CFUs of each strain under H_2O_2+ and H_2O_2− conditions was represented as a relative value (%) against the respective baseline under H_2O_2− conditions. CFUs for *S. marinintestina* strain IK-1 were much higher than those for strain IK-1Δ8 at any concentration of H_2O_2 (Fig. 1).

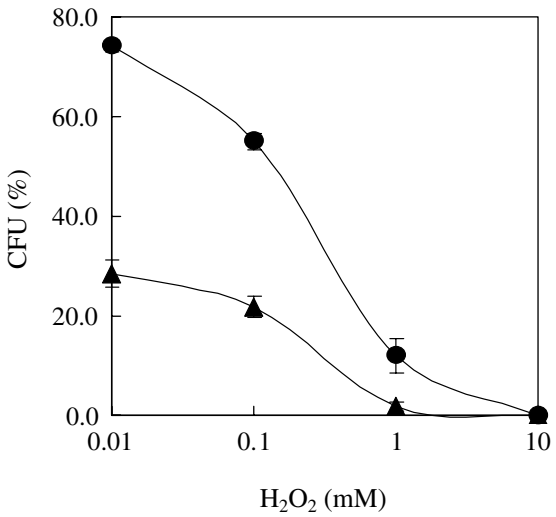

Fig. 1. Effects of H_2O_2 on the colony-forming ability of *Shewanella marinitestina* IK-1 and its EPA-deficient mutant IK-1Δ8. Cells of strain IK-1 (●) and strain IK-1Δ8 (▲) were suspended in phosphate-buffered saline. After incubated for 30 min in the presence of 0.01, 0.1, 1, and 10 mM H_2O_2 under non-growth conditions, the number of CFUs was determined. The baseline value of each strain was that of cells treated without H_2O_2. The CFU of *S. marinintestina* strains IK-1 and IK-1Δ8 was $7.5 \pm 0.3 \times 10^9$ ml of culture^{-1} and $7.2 \pm 0.3 \times 10^9$ ml of culture^{-1}, respectively, at each baseline. The data indicated are means ± S.E. for three independent experiments.

3.2. Effects of H_2O_2 on catalase activity and carbonyl contents in S. marinintestina strains IK-1 and IK-1Δ8

The catalase activity was almost the same in both *S. marinintestina* strains IK-1 and IK-1Δ8 and it was not affected by the presence of H_2O_2 (data not shown). It should be noted that the baseline catalase activity (0.18 U mg protein^{-1}) of *S. marinintestina* IK-1 was remarkably low, being only one tenth of that of *E. coli* and other bacteria such as *Bacillus subtilis* and *Vibrio parahaemolyticus* [16].

When bacterial cells are treated with H_2O_2, various proteins such as elongation factor G [18] or glyceraldehyde-3-phosphate dehydrogenase [19] are carbonylated, leading to a growth arrest. The baseline level of protein carbonyls in *S. marinintestina* strains IK-1 and IK-1Δ8 was approximately 80 nmol mg protein^{-1} (Table 1). In *S. marinintestina* IK-1, levels of protein carbonyls did not change notably after incubation with 0.01 mM H_2O_2 for 30 min, while for strain IK-1Δ8, levels of protein carbonyl increased slightly from 83.1 ± 1.3 nmol mg protein^{-1} to 94.1 ± 1.9 nmol mg protein^{-1} for 30 min under H_2O_2+ conditions. However, no change was observed in either strain under H_2O_2− conditions.

Table 1
Effects of H_2O_2 on the protein carbonyl contents of *Shewanella marinitestina* IK-1[a] and its EPA-deficient mutant IK-1Δ8[a]

	Protein carbonyl (nmol mg protein^{-1})		
	H_2O_2-		H_2O_2+
	0[b]	30[b]	30[b]
IK-1	78.6 ± 0.9	82.2 ± 5.5	84.1 ± 5.0
IK-1Δ8	83.1 ± 1.3	84.6 ± 3.0	94.1 ± 1.9

The data indicated are means ± S.E. for three independent experiments.
[a]Cells were treated with 0.01 mM H_2O_2.
[b]Time (min).

3.3. Effects of H_2O_2 on amount of *S. marinintestina* strains IK-1 and IK-1Δ8 cells and their fatty acid composition

The baseline weight of dry cells from 1 ml of culture was 220 ± 30 μg and 190 ± 30 μg for *S. marinintestina* strains IK-1 and IK-1Δ8, respectively. When cells were treated with 0.01 mM H_2O_2 for 30 min a significant decrease in the dry cell weight per culture were observed only for strain IK-1Δ8 (Table 2). Little changes were observed in either strain under H_2O_2- conditions. Similarly the amount of fatty acid extracted from H_2O_2-treated cells of *S. marinintestina* IK-1Δ8 was much lesser than that of *S. marinintestina* IK-1 (data not shown).

The major fatty acids in *S. marinintestina* IK-1 cells were dodecanoic acid (10.9 ± 1.1%), tetradecanoic acid (15.1 ± 0.3%), palmitoleic acid (22.4 ± 0.6%), and EPA (17.3 ± 0.4%). Under the same condition, no EPA was detected and levels of palmitoleic acid significantly increased to 49.0 ± 0.5% in cells of strain IK-1Δ8. Treating both strains with H_2O_2 had little effect on their fatty acid composition (data not shown).

S. marinintestina strains IK-1 and IK-1Δ8 had phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), accounting for approximately 70 mol% and 25 mol%, respectively, of total lipids. In strain IK-1 all of EPA was detected in PE and PG.

3.4. Extracellular and intracellular concentrations of H_2O_2 in *S. marinintestina* strains IK-1 and IK-1Δ8

The extracellular and intracellular concentrations of H_2O_2 were also determined for *S. marinintestina* strains IK-1 and IK-1Δ8 using cells incubated under bacteriostatic conditions in the presence of 0.1 mM H_2O_2 and where no H_2O_2 was present. Cells were treated with 0.1 mM H_2O_2 in consideration of the detection limit of H_2O_2 by the titanium-based method [12]. The extracellular levels of H_2O_2 for both strains decreased slightly and gradually over time (Fig. 2A). The rate of decrease was essentially the same for cells of *S. marinintestina*strains IK-1 and IK-1Δ8. Negligible concentrations of H_2O_2 were consistently detected in cells of both strains where no H_2O_2 was present (Fig. 2A). The intracellular concentration of H_2O_2 increased over time for both strains under H_2O_2 conditions. The H_2O_2 concentrations were 20.8 ± 4.2 μM for strain IK-1 and 39.4 ± 3.1 μM for strain IK-1Δ8 cells, 30 min after the addition

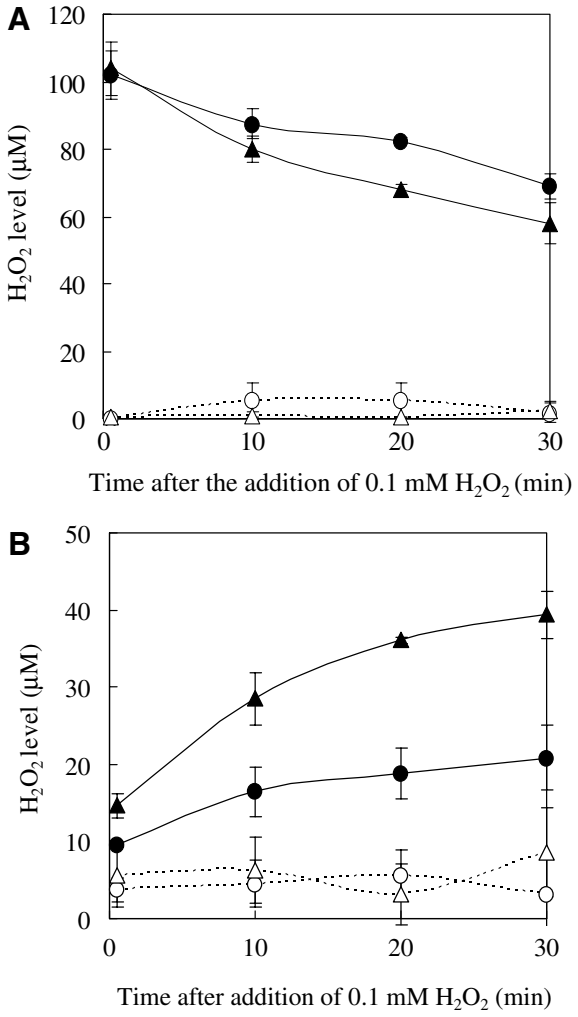

Fig. 2. Changes of extracellular (A) and intracellular (B) concentrations of H_2O_2 in *Shewanella marinitestina* strains IK-1 and IK-1Δ8 cells that were treated with and without H_2O_2 under non-growth conditions. Portions of cell suspensions were withdrawn at various time intervals and assayed to determine extracellular and intracellular H_2O_2 concentrations. △, strain IK-1Δ8 under H_2O_2- conditions; ▲, IK-1Δ8 under H_2O_2+ conditions; ○, IK-1 under H_2O_2- conditions; ●, IK-1 under H_2O_2+ conditions. Cells were incubated at 15 °C. The data indicated are means ± S.E. for three independent experiments.

Table 2
Effects of 0.01 mM H_2O_2 on the dry cell weight of *Shewanella marinitestina* IK-1 and its EPA-deficient mutant IK-1Δ8 that had been treated with and without H_2O_2 under bacteriostatic conditions

	Dry cell weight (μg ml cell culture^{-1})			
	IK-1		IK-1Δ8	
	H_2O_2-	H_2O_2+	H_2O_2-	H_2O_2+
0 time	220 ± 30 (100.0)		190 ± 30 (100.0)	
30 min	210 ± 0.0 (95.4)	210 ± 10 (94.5)	200 ± 20 (103.1)	140 ± 20 (71.2)

Relative values are in parenthesis. The data indicated are means ± S.E. for three independent experiments.

of H_2O_2 (Fig. 2B). The intracellular concentration of H_2O_2 in cells of *S. marinintestina* strain IK-1 was approximately one-quarter of that in cells of strain IK-1Δ8.

4. Discussion

In this study, the antioxidative function of EPA was demonstrated in the marine bacterium *S. marinintestina* IK-1 that inherently produces EPA and in its EPA-deficient mutant (strain IK-1Δ8). *S. marinintestina* IK-1 produced EPA at approximately 17% of total fatty acids at 15 °C. *S. marinintestina* IK-1 completely lost the capacity to synthesize EPA after the insertional inactivation mutagenesis of the *pfaD* gene. The lack of EPA in strain IK-1Δ8 was maintained after repeated cultivations in a medium without antibiotics.

Previously we presented data based on *E. coli* recombinant systems showing that EPA in the cell membrane exerted an antioxidative effect on exogenously added H_2O_2. Although the molecular mechanism of this antioxidative function of EPA is unknown, it has been speculated previously that the inherent molecular properties of EPA (as acyl constituents of phospholipids) allow it to form a more highly packed structure [20] than most other phospholipids so that it can inhibit the entry of H_2O_2 through the cell membrane. Considering that polyunsaturated fatty acids like EPA and docosahexaenoic acid are cellular molecules with the greatest susceptibility to oxidation [21], this shielding function of EPA against exogenous H_2O_2 is a unique mechanism. This mechanism operated against other oxidants such as *t*-butyl hydroperoxide (analog of hydrogen peroxide and not degraded by catalase) (unpublished result).

The ability of EPA to shield the cell membrane against exogenous H_2O_2 was also present in bacteria that naturally produced EPA. The CFU counting method (Fig. 1) showed that *S. marinintestina* IK-1 that produced EPA was more resistant to H_2O_2 than its EPA-deficient mutant strain IK-1Δ8. The higher resistance of *S. marinintestina* IK-1 to H_2O_2 was exemplified by the finding that lower levels of protein carbonyls were detected in *S. marinintestina* IK-1 (with EPA) than in strain IK-1Δ8 (without EPA) (Table 1), and that the recovered amount of cells (dry cell weight) (Table 2) and then simultaneously that of fatty acids was much higher from cultures of strain IK-1 than from cultures of strain IK-1Δ8 (data not shown). The latter observation suggests that more significant cell breakage occurred in strain IK-1Δ8 than in strain IK-1 and that EPA in cell membrane phospholipids function to prevent the entry of H_2O_2 from the external medium. The cell breakage was at a level of approximately 30% in strain IK-1Δ8 that had been treated with 0.01 mM H_2O_2 (Table 2), where CFU of this strain was much more significantly reduced (approximately 70%; Fig. 1). These results suggest that a viable but non-culturable state might have been induced in some of H_2O_2-treated strain IK-1Δ8 cells, as was the case of H_2O_2-treated *E. coli* [1]. Although the shielding mechanism of the cell membrane is largely unknown, we speculate the involvement of the structural hindrance of EPA-containing phospholipids, whose structure is more compact and prohibits the entry of H_2O_2 from outside.

Levels of protein carbonyls in non-growth cells of *S. marinintestina* strains IK-1 and IK-1Δ8 before treatment with H_2O_2 were approximately 80 nmol mg protein^{-1}. Only slightly higher values were obtained for the mutant cells (Table 1). These values were significantly higher than those measured in catalase-deficient *E. coli* UM2 transformant cells, both with EPA and without EPA, in which levels of protein carbonyls determined under the same conditions were approximately 0.2 nmol mg protein^{-1}. The value was increased to approximately 0.5 nmol mg protein^{-1} and 0.75 nmol mg protein^{-1} for cells with EPA and those without EPA, respectively [2]. This was despite *E. coli* UM2 having no catalase or other notable H_2O_2-decomposing activity and *E. coli* UM2 transformants were treated with 0.3 mM H_2O_2, whereas *S. marinintestina* strains IK-1 and IK-1Δ8 were treated with 0.01 mM H_2O_2. All of these observations suggest that the cellular proteins of *S. marinintestina* strains might be much more susceptible to oxidation than the cellular proteins of *E. coli*. It is known that the marine bacteria *Vibrio rumoiensis* and *V. parahaemolyticus* are considerably more sensitive to exogenous H_2O_2 than *E. coli* and *B. subtilis* [22]. However, it is unknown whether such a characteristic is common in marine bacteria. To determine this would require biochemical and molecular testing of the susceptibility of proteins to oxidants.

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